CONCISE COMMUNICATION

Evidence for efflux pumps, other than PmrA, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*

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Fluoroquinolone resistance in pneumococci is known to be associated with the efflux pump, PmrA. However, there may be other efflux systems that also cause drug resistance. Two types of mutants were studied. The efflux phenotype from mutants selected by sub-MIC levofloxacin or gemifloxacin was transformed into R6. These transformants did not show increased *pmrA* transcripts in Northern blots; insertional inactivation of *pmrA* in the transformants did not abolish the efflux phenotype. A second set of efflux phenotype mutants was selected in R6:*cat* by ethidium bromide but not by norfloxacin; accumulation of ethidium bromide in the one among these mutants studied was reduced in comparison to its parent. This evidence suggests that systems other than PmrA can contribute to efflux-mediated resistance in pneumococci.

Keywords Pneumococcus, efflux, quinolone, PmrA

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Multidrug efflux resistance is well described in *Streptococcus pneumoniae* [1–3]. It is associated with a reduction in the susceptibility to some fluoroquinolones, such as norfloxacin and ciprofloxacin, as well as to several unrelated dyes (e.g. acriflavine and ethidium bromide) and disinfectants (e.g. cetrimide). In common with pumps of the major facilitator and ABC superfamilies, that described in pneumococci is inhibited by reserpine. Increased expression of a 399 amino acid protein (PmrA) of the major facilitator superfamily of efflux pumps has been implicated in multidrug efflux resistance in pneumococci [4].

In other Gram-positive bacteria, multiple efflux systems have been shown to occur in the same organism. For example, in *Bacillus subtilis* the overexpression of the efflux pumps Bmr or Blt causes a reduction in the susceptibility to several compounds, including norfloxacin and ethidium bromide [5]. Similarly, in *Staphylococcus aureus*, at least two multidrug efflux pumps have been shown to exist [6].

As several multidrug efflux systems can occur in the same organism and often have overlapping substrates, we undertook this study to determine whether efflux pumps other than PmrA are likely to contribute to multidrug resistance in pneumococci. Since bacteria may have more than one such efflux pump, we have studied mutants derived by two different means to see if these mutants would have different efflux pumps overexpressed.

The first mutants we studied, LEV1 and GEM11, had been selected from clinical isolates by serial subculture in the presence of low levels of levofloxacin and gemifloxacin, respectively [7,8]. Both mutants had previously been shown to have a phenotype consistent with efflux-mediated fluoroquinolone resistance [7,8], in addition to topoisomerase II mutations. To study the efflux resistance in these strains apart from the effects of their topoisomerase mutations, the susceptible laboratory strain R6 was transformed by a standard method [4] using chromosomal DNA extracted from LEV1 and GEM11 as donor DNA. Transformants were selected with ethidium bromide (4 mg/L), which is a pump substrate and has been used to select efflux pump mutants in pneumococci [1]. To control for spontaneous

ethidium bromide-resistant mutants, an aliquot of R6 cells (which was not exposed to transforming DNA) was taken through the transformation procedure in parallel; no spontaneous ethidium bromide-resistant mutants were detected in this control.

The transformation of strain R6 with chromosomal DNA from efflux mutants LEV1 and GEM11 yielded transformants selected with ethidium bromide (transformation frequencies 2×10^{-5} and 7×10^{-5}, respectively; *parC* mutant donor control 5×10^{-5} selected on norfloxacin 4 mg/L). A standard agar dilution method [9] was employed to determine the susceptibility of transformants to several fluoroquinolones and known efflux pump substrates; the effect of the efflux pump inhibitor, reserpine (10 mg/L), on the norfloxacin minimum inhibitory concentration (MIC) was also tested as previously [2]. Phenotypically typical transformants, R6LEV1 and R6GEM11, were chosen for further study. Table 1 shows the susceptibility of these two transformants. Both showed a two- to four-fold rise in the MIC of norfloxacin compared with R6, which was reduced four- to eight-fold in the presence of reserpine. The MICs of ethidium bromide and acriflavine were increased four-to-16-fold. The MICs of moxifloxacin and gemifloxacin were not significantly increased. The MICs of R6LEV1 and R6GEM11 are consistent with an efflux phenotype [2], since they show low-level resistance to unrelated drugs. Insertional inactivation of *pmrA* with *cat* [4] in R6LEV1 and R6GEM11 did not alter this phenotype. This indicates that the efflux phenotype in R6LEV1 and R6GEM11 is independent of PmrA.

To see whether or not the efflux phenotype transformants R6LEV1 and R6GEM11 had increased expression of *pmrA*, Northern blotting was performed on total RNA on pneumococci grown to mid-log phase. A derivative of R6 with increased expression of *pmrA* (R6N [4]) was used as a positive control for this blotting. Northern blot analysis of R6LEV1 and R6GEM11 for *pmrA* did not show increased transcription compared with the parent strain R6. These results indicate that in these strains, the efflux phenotype was independent of increased transcription of *pmrA*.

The second set of mutants we studied were those arising from ethidium bromide selection of a strain (R6:*cat*) in which *pmrA* was insertionally inactivated. A similar approach has been used by Kaatz et al. [6] in *Staphylococcus aureus* to demonstrate the presence of a multidrug efflux system

Table 1 Susceptibility of *Streptococcus pneumoniae* strains to fluoroquinolones and efflux pump substrates

Organism	MIC (mg/L)									
	Nor	Nor R+	Cip	Gem	Mox	Ebr	Ari	Cet	Tet	TPP	Chlo
Transformants of R6											
R6 (susceptible parent)	4	2	1	0.03	0.12	2	4	8	0.25	64	2
R6LEV1	16	2	2	0.06	0.25	16	16	16	0.5	64	2
R6GEM11	8	2	2	0.03	0.12	32	16	16	0.5	64	2
Mutants selected from R6:*cat* with ethidium bromide											
R6:*cat* (*pmrA::cat*; parent)	2	2	1	0.03	0.12	1	4	8	0.25	64	32
R6:*cat*-1 (ethidium bromide mutant)	16	4	4	0.03	0.25	16	16	16	0.5	64	32
R6:*cat*-2 (ethidium bromide mutant)	16	4	2	0.03	0.12	16	16	16	0.5	64	32

Nor, norfloxacin; Cip, ciprofloxacin; Gem, gemifloxacin; Mox, moxifloxacin; Ebr, ethidium bromide; Ari, acriflavine; Cet, cetrimide; Tet, tetracycline; TPP, tetraphenylphosphonium bromide; Chlo, chloramphenicol; R+, with reserpine (mg/L).

other than NorA. Insertional inactivation was done in the standard laboratory strain R6, with a chloramphenicol resistance cassette (*cat*), by the method previously described [4]. To select for mutants of R6:*cat*, approximately 10^9 CFU of *S. pneumoniae* strain R6:*cat* were inoculated on Columbia blood agar containing either norfloxacin or ethidium bromide at 1×, 2×, 4× or 8× their respective MICs of 2 mg/L and 1 mg/L. After incubation for 3 days at 35–37 °C in 4–6% CO_2, representative mutants were subcultured on Columbia blood agar containing 8 mg/L chloramphenicol.

Of 68 norfloxacin-selected mutants examined, none showed a greater than two-fold reduction in their susceptibility to norfloxacin in the presence of reserpine. Since the reduction in their susceptibility, as determined by agar dilution testing, was not significant (i.e. $\leq$ 2-fold increase in MIC), they were not further studied. However, two mutants (R6:*cat*-1 and R6:*cat*-2) selected with ethidium bromide at a concentration of 8 × MIC were found to be presumptive efflux mutants. Both showed an eight-fold rise in MIC of norfloxacin compared with the parent strain (R6:*cat*), which was reduced four-fold in the presence of reserpine. The mutants also showed significantly raised MICs to ethidium bromide and acriflavine (Table 1) compared with the parent.

It is of interest that efflux mutants could only be selected from R6:*cat* with ethidium bromide; none were selected with norfloxacin. In other studies using different strains, both ethidium bromide and norfloxacin have been shown to readily select efflux mutants from *S. pneumoniae* [1,2]. It is possible that the inability of norfloxacin to select efflux mutants from R6:*cat* merely reflects differences between strains, or that ethidium bromide is a more suitable selection agent for efflux mutants. The mutants that we selected with norfloxacin probably represent topoisomerase mutants: their susceptibility to norfloxacin was not significantly affected by reserpine, nor was their susceptibility to ethidium bromide reduced.

To further confirm the efflux phenotype in the ethidium bromide-selected mutants, ethidium bromide accumulation was determined in R6:*cat* and R6:*cat*-2, as previously [2]. Mutant R6:*cat*-2 accumulated less ethidium bromide than the parent strain R6:*cat* (Figure 1). After 10 min, the accumulation was 26% lower in the mutant than in the parent (Student's *t*-test, $P < 0.05$).

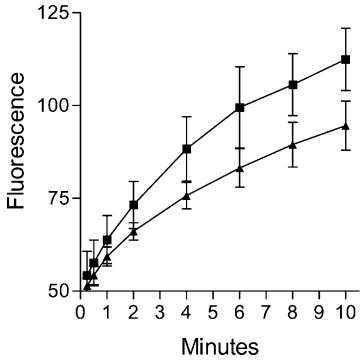

Figure 1 Accumulation of ethidium bromide by *Streptococcus pneumoniae* strain R6:*cat* (parent; squares) and R6:*cat*-2 (mutant; triangles). Each point is the mean of three individual experiments; error bars represent ± standard deviation. Fluorescent emission at 600 nm is proportional to accumulation of ethidium bromide.

Finally, Southern blot analysis of chromosomal DNA (digestion with *Pst*I or *Hin*dIII and probed for *pmrA* and *cat*) from R6:*cat* and its ethidium bromide-selected efflux mutants was performed as previously described [4]. This blotting confirmed (data not shown) the presence of a single copy of *pmrA* on the chromosome of R6:*cat*, R6:*cat*-1 and R6:*cat*-2. It also showed that *cat* was within *pmrA* for the three strains. These findings indicate that in the ethidium bromide-selected mutants, the efflux phenotype was unlikely to be due to expression of *pmrA* or the effects of *cat* insertion elsewhere in the chromosome.

We have found multidrug efflux-mediated resistance in mutants R6LEV1 and R6GEM1 that is not associated with increased expression of *pmrA*. We have also selected multidrug efflux mutants (R6:*cat*-1 and R6:*cat*-2) in the presence of an inactivated copy of *pmrA*. This is good evidence that systems other than PmrA can contribute to efflux-mediated resistance in pneumococci.

The phenotypes that we have described do not cause significant resistance to fluoroquinolones, such as gemifloxacin and moxifloxacin, with anti-pneumococcal activity. However, it is of interest that levofloxacin and gemifloxacin may select for such phenotypes. In addition, the pump(s) system(s) indicated by our experiments may be of relevance to other antibiotics now and in the future, and will help to define efflux pump systems in pneumococci. Genome sequencing of strains TIGR4 [10] and R6 [11] indicates a large number of putative multifacilitator and ABC

superfamily efflux pump genes. In this latter group of pumps, there are approximately 70 without assigned function. The role that these putative pump genes play in non-PmrA-mediated drug resistance needs further study. It is likely that the efflux pumps found in pneumococci, as in other bacteria, will have overlapping substrate specificity and complex regulation of their expression [5,6].

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REFERENCES

1. Baranova NN, Neyfakh AA. Apparent involvement of a multidrug transporter in the fluoroquinolone resistance of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1997; 41: 1396–8.
2. Brenwald NP, Gill MJ, Wise R. Prevalence of a putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1998; 42: 2032–5.
3. Zeller V, Janoir C, Kitzis M, Gutmann L, Moreau NJ. Active efflux as a mechanism of resistance to ciprofloxacin in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1997; 41: 1973–8.
4. Gill MJ, Brenwald NP, Wise R. Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1999; 43: 187–9.
5. Ahmed M, Lyass L, Markham PN, Taylor SS, Vazquez-Laslop N, Neyfakh AA. Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. *J Bacteriol* 1995; 177: 3904–10.
6. Kaatz GW, Seo SM, O'Brien L, Wahiduzzaman M, Foster TJ. Evidence for the existence of a multidrug efflux transporter distinct from NorA in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2000; 44: 1404–6.
7. Davies TA, Pankuch GA, Dewasse BE, Jacobs MR, Appelbaum PC. In vitro development of resistance to five quinolones and amoxicillin–clavulanate in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1999; 43: 1177–82.
8. Nagai K, Davies TA, Dewasse BE, Jacobs MR, Appelbaum PC. In vitro selection of resistant mutants of *Streptococcus pneumoniae* to gemifloxacin, trovafloxacin, ciprofloxacin, gatifloxacin and moxifloxacin [abstract 744]. In: *Program and Abstracts of the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada*. Washington, DC: American Society for Microbiology, 2000: 79.
9. Working Party of the British Society of Antimicrobial Chemotherapy. A guide to sensitivity testing. *J Antimicrob Chemother* 1991; 27(suppl D): 22–30.
10. Tettelin H, Nelson KE, Paulsen IT *et al.* Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* 2001; 293: 498–506.
11. Hoskins J, Alborn WE Jr, Arnold J *et al*. Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J Bacteriol* 2001; 183: 5709–17.